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The Hexosamine Biosynthesis Pathway Is Essential for Pancreatic Beta Cell Development*

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Pancreatic exocrine and endocrine cells develop during embryonic life from endodermal progenitors. This process depends on activation of a hierarchy of transcription factors. Although information is available regarding the mesodermal signals controlling pancreas development, little is known about the role of environmental factors such as nutrients, including glucose, that also may impact development. Previously, we showed that glucose plays an important and specific role in beta cell development by activating the transition of Neurogenin3-positive endocrine progenitors into beta cells. Here, we examined the implication of glucose metabolism and more precisely the role of the hexosamine biosynthesis pathway (HBP) to understand the mechanisms by which glucose regulates beta cell development. We have established an *in vitro* model of endocrine and exocrine cells development from embryonic day 13.5 rat pancreases in a manner that replicates *in vivo* pancreas development perfectly. Using this model, we tested the effect of selective inhibitors and activators of the HBP and found that the HBP has a modest effect on cell proliferation and exocrine cell differentiation. On the other hand, beta cell development is tightly controlled by the HBP. Specifically, HBP activators increase beta cell development, whereas inhibitors repress such development. Importantly, both the HBP and glucose control the same steps in beta cell development.

The mature pancreas contains two types of tissues: exocrine tissue, which is composed of acinar cells that secrete digestive enzymes into the intestine via a branched network of ductal cells, and endocrine islets of Langerhans cells that produce hormones such as insulin (beta cells), glucagon (alpha cells), somatostatin (delta cells), pancreatic polypeptide cells, and ghrelin (epsilon cells) (1). The pancreas originates from the dorsal and ventral regions of foregut endoderm directly posterior to the stomach. The first indication of pancreatic morphogenesis occurs in mice at embryonic day (E)[3] 8.5 (E9.5 in rat), when the endoderm evaginates to form buds (2, 3). Subsequently, the mesenchyme condenses around the underlying endoderm, and the epithelial buds grow in size, whereas exocrine and endocrine cells differentiate (4).

During development, the endodermal region committed to form the pancreas initially expresses the transcription factor *Pdx-1* (pancreatic-duodenal homeobox 1), a marker of pancreatic progenitors also expressed in mature beta cells (5–7). The basic helix-loop-helix factor *Ngn3* (Neurogenin3) is then expressed in epithelial pancreatic progenitor cells prior to endocrine differentiation (8, 9). Thus, *Ngn3* is a valuable marker for monitoring pancreatic endocrine cell differentiation. NGN3 controls the expression of *NeuroD*, which is another member of the basic helix-loop-helix transcription factor family (10). Mice that lack *NeuroD* exhibit strongly perturbed islet development, demonstrating the importance of this transcription factor for development of a normal endocrine pancreas (11).

Pancreas development is known to be controlled by signals derived from tissues that contact the endodermal region that gives rise to the pancreas (1), such as the notochord (12), the dorsal aorta (13), and the pancreatic mesenchyme (14). Interestingly, animal models of intrauterine growth retardation have also demonstrated the importance of uterine nutrition on pancreas development (15, 16), but less is known regarding the importance of nutrients in controlling its development. Notably, these studies were performed *in vivo*, limiting the ability to assess the molecular processes that restrict pancreatic development. Moreover, an *in vivo* approach does not permit detailed analysis of the precise effects of specific nutrients on pancreatic development.

Previously, we developed an *in vitro* model of beta cell development using rat embryonic pancreas that perfectly mimics *in vivo* pancreatic development (17). With this model, we have shown that glucose controls beta cell development by activating expression of *NeuroD*, which is a direct target of NGN3 (18). In this study, we built upon these findings and attempted to determine how glucose exerts such effects on beta cell development. Typically, glucose is transported inside the cell and phosphorylated into glucose-6-phosphate. Next, it enters the glycolysis pathway to provide energy. However, 2–5% of glucose is directed into the hexosamine biosynthetic pathway (HBP) to promote protein glycosylation (19). Glutamine:fructose-6-

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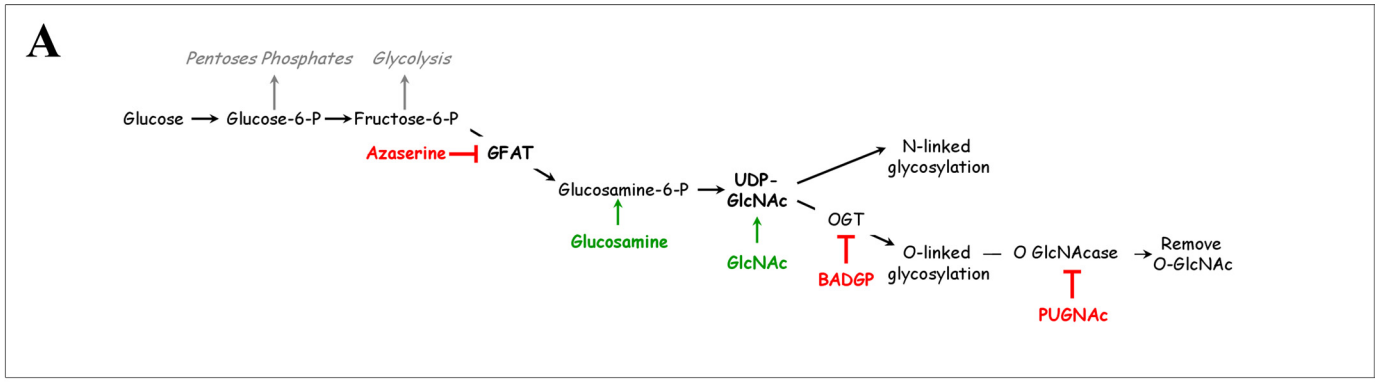
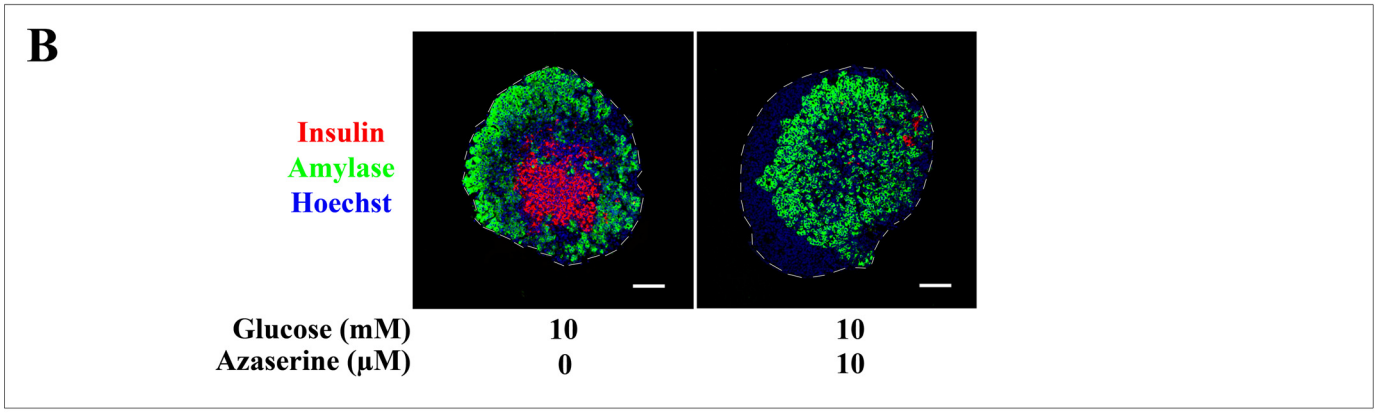
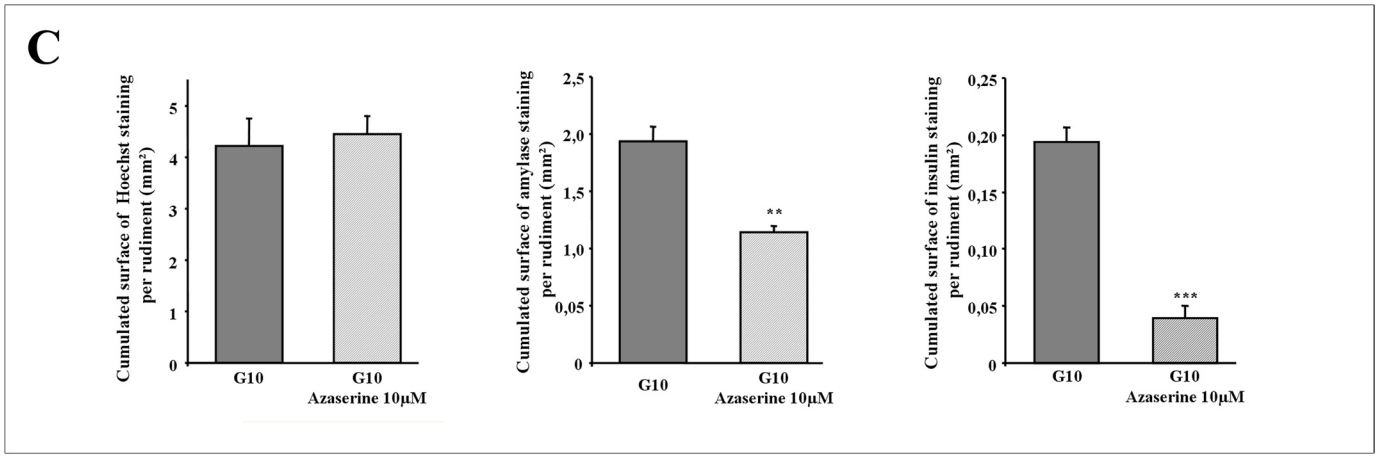
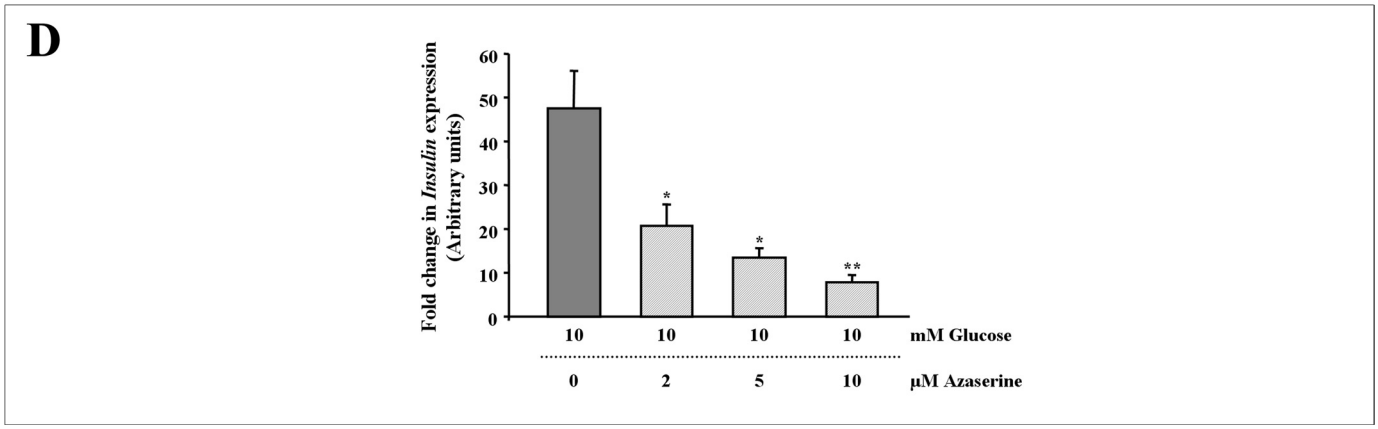
[3] The abbreviations used are: E*n*, embryonic day *n*; HBP, hexosamine biosynthetic pathway; GFAT, glutamine:fructose-6-phosphate amidotransferase; *O*-GlcNAcase, β-D-*N*-acetylglucosaminidase; OGT, *O*-linked β-*N*-acetylglucosaminyl transferase; BADGP, benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside; FGF, fibroblast growth factor receptor; PUGNAc, *O*-2-acetamido-2-deoxy-D-glucopyranosylidene amino *N*-phenylcarbamate; BrdUrd, bromodeoxyuridine.


A
Pentoses Phosphates
Glycolysis
Glucose
Glucose-6-P
Fructose-6-P
Azaserine
GFAT
Glucosamine-6-P
UDP-GlcNAc
N-linked glycosylation
Glucosamine
GlcNAc
OGT
BADGP
O-linked glycosylation
O GlcNAcase
Remove O-GlcNAc
PUGNAc


B
Insulin
Amylase
Hoechst
Glucose (mM) 10 10
Azaserine (µM) 0 10


C
Cumulated surface of Hoechst staining per rudiment (mm²)
G10
G10 Azaserine 10µM
Cumulated surface of amylase staining per rudiment (mm²)
**
Cumulated surface of insulin staining per rudiment (mm²)
***


D
Fold change in Insulin expression (Arbitrary units)
*
*
**
10 10 10 10 mM Glucose
0 2 5 10 µM Azaserine

phosphate amidotransferase (GFAT) is the first enzyme in the HBP. GFAT transfers the amide group from glutamine to fructose-6-phosphate to form glucosamine-6-phosphate, a precursor of uridine diphosphate-GlcNAc. The latter is the major substrate for protein *N*- and *O*-glycosylation. When *N*-glycosylation is constitutive, *O*-glycosylation is a process (similar to phosphorylation) regulated by *O*-linked β-*N*-acetylglucosamine transferase (OGT) and β-D-*N*-acetylglucosaminidase (O-GlcNAcase), which are responsible for *O*-glycosylation and de-*O*-glycosylation mechanisms, respectively (reviewed in Refs. 20 and 21). Here, we perturbed the HBP with specific inhibitors and activators using our *in vitro* model of beta cell development from pancreatic progenitors and demonstrated that the HBP controls specific steps of pancreatic beta cell development.

EXPERIMENTAL PROCEDURES

Animals and Dissection of Dorsal Pancreatic Rudiments—Pregnant Wistar rats were purchased from CERJ (Le Genest, St. Isle, France). The first day post-coitum was designated embryonic day 0.5 (E0.5). Pregnant female rats at 13.5 days of gestation were killed by CO_2 asphyxiation in compliance with the French Animal Care Committee's guidelines. Dorsal pancreatic buds from E13.5 rat embryos were dissected as described previously (22).

Organ Culture, Activator and Inhibitor Treatments, and BrdUrd Incorporation—Pancreases were laid on 0.45-μm filters (Millipore, St-Quentin-en-Yvelines, France) at the air-medium interface in Petri dishes containing RPMI 1640 (Lonza, Basel, Switzerland) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), HEPES (10 mmol/liter), nonessential amino acids (1×; Invitrogen), and 10% heat-inactivated calf serum (HyClone, Logan, UT) (17). The cultures were maintained at 37 °C in humidified 95% air, 5% CO_2. D-glucose, GlcNAc, azaserine, glucosamine, benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP; Sigma-Aldrich), 3-(2-adamantanylethyl)-2-[(4-chlorophenyl)-azamethylene]-4-oxo-1,3-thiazaperhyd roine-6-carboxylic acid (ST045849; TimTec, Newark, DE), and *O*-2-acetamido-2-deoxy-D-glucopyranosylidene amino *N*-phenylcarbamate (PUGNAc; Toronto Research Chemical, North York, Canada) were used at the indicated concentrations. The medium was changed every other day. For cell proliferation assays, 10 μM BrdUrd (Sigma-Aldrich) was added to the medium during the last hour of culture.

Immunohistochemistry—The tissues were fixed in 10% formalin, pre-embedded in low gelling agarose, and embedded in paraffin. All of the sections (4 μm thick) of each pancreatic explant were collected and processed for immunohistochemical analysis as described previously (23, 24). Antibodies were used at the following dilutions: mouse anti-insulin, 1/2,000; rabbit anti-amylase, 1/300 (both from Sigma-Aldrich); rabbit anti-PDX-1 (25), 1/1,000; mouse anti-BrdUrd (Amersham Biosciences), 1/4; and rabbit anti-NGN3 (18), 1/1,000. The fluorescent secondary antibodies used included fluorescein anti-rabbit 1/200 and Texas Red anti-mouse antibody 1/200 (both from Jackson Immunoresearch, Suffolk, UK) and Alexa Fluor 488 anti-rabbit antibody 1/400 (Invitrogen). The nuclei were stained in blue with Hoechst 33342 (0.3 μg/ml; Invitrogen). NGN3 detection was performed as previously described (18) using the Vectastain elite ABC kit (Vector Laboratories, Burlingame, Canada). Photographs were taken using a fluorescence microscope (Leitz DMRB; Leica, Rueil-Malmaison, France).

Quantification—All of the sections from each pancreatic rudiment were digitized using cooled, three-charge coupled device cameras (C5810 or C7780; Hamamatsu, Middlesex, NJ). On every image, the surface area of each staining was quantified using ImageJ 1.34s and summed to obtain the total surface area/explant in mm^2 as previously described (18, 26). To quantify proliferation of early PDX-1-positive pancreatic progenitors, we counted the frequency of BrdUrd-positive nuclei among 2,000 early PDX-1-positive progenitors/rudiment. To quantify the absolute numbers of NGN3-positive endocrine progenitors, all sections of each pancreatic rudiment were digitized, and on every section, the number of NGN3-positive cells was counted (18, 26). At least four explants grown under each set of culture conditions were analyzed. The results are expressed as the means ± S.E.

RNA Extraction and Real Time PCR—Total RNA was extracted from pools of three pancreases using an RNeasy Microkit (Qiagen) and reverse transcribed using Superscript reagents (Invitrogen). Real time PCR was performed with the 7300 fast real time PCR system (Applied Biosystems, Paris, France). Oligonucleotide sequences are available upon request. Peptidylpropyl isomerase A was used as endogenous control. E16.5 pancreas cDNA was used as calibrator sample. The data were analyzed by the comparative cycle threshold method and presented as the fold change in gene expression normalized to an E16.5 calibrator that equals a value of one (18). At least four pools of explants were analyzed per condition, and the results are expressed as the means plus S.E.

Statistics—The results are expressed as the means ± S.E. Statistical significance was determined using Student's *t* test when only two sets of data were compared. For larger analysis, a nonparametric Kurskal & Wallis test was performed, followed by a Mann-Whitney test.

RESULTS

Inhibition of HBP Affects Pancreas Development—When E13.5 rat pancreases are dissected and cultured on filters floating at the air-medium interface, acinar and endocrine cells develop in a manner that replicates *in vivo* pancreatic development (17). To define the role of HBP in beta cell differentiation, we used this rat pancreas model to examine the impact of treatment with azaserine, a competitive inhibitor of GFAT, the first rate-limiting enzyme of the HBP pathway (Fig. 1*A*). Pancreases

FIGURE 1. **Azaserine treatment prevents beta cell development.** *A*, schematic diagram of the HBP. The activators and inhibitors used in this study are colored in *green* and *red*, respectively. *B*, immunohistological analysis of pancreases after 7 days of culture in the presence of 10 mM glucose with or without 10 μM azaserine. Insulin was revealed in *red*, and amylase is in *green*. The nuclei are depicted in *blue* by Hoechst stain. *Scale bar*, 100 μm. *C*, quantification of the absolute surface area occupied by Hoechst-, amylase-, and insulin-positive cells after 7 days of culture with or without azaserine. *D*, real time PCR quantification of *Insulin* mRNA after 7 days of culture in the presence of glucose 10 mM at increasing concentrations of azaserine. The values are the means ± S.E. of five independent experiments and were compared with G10 condition. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

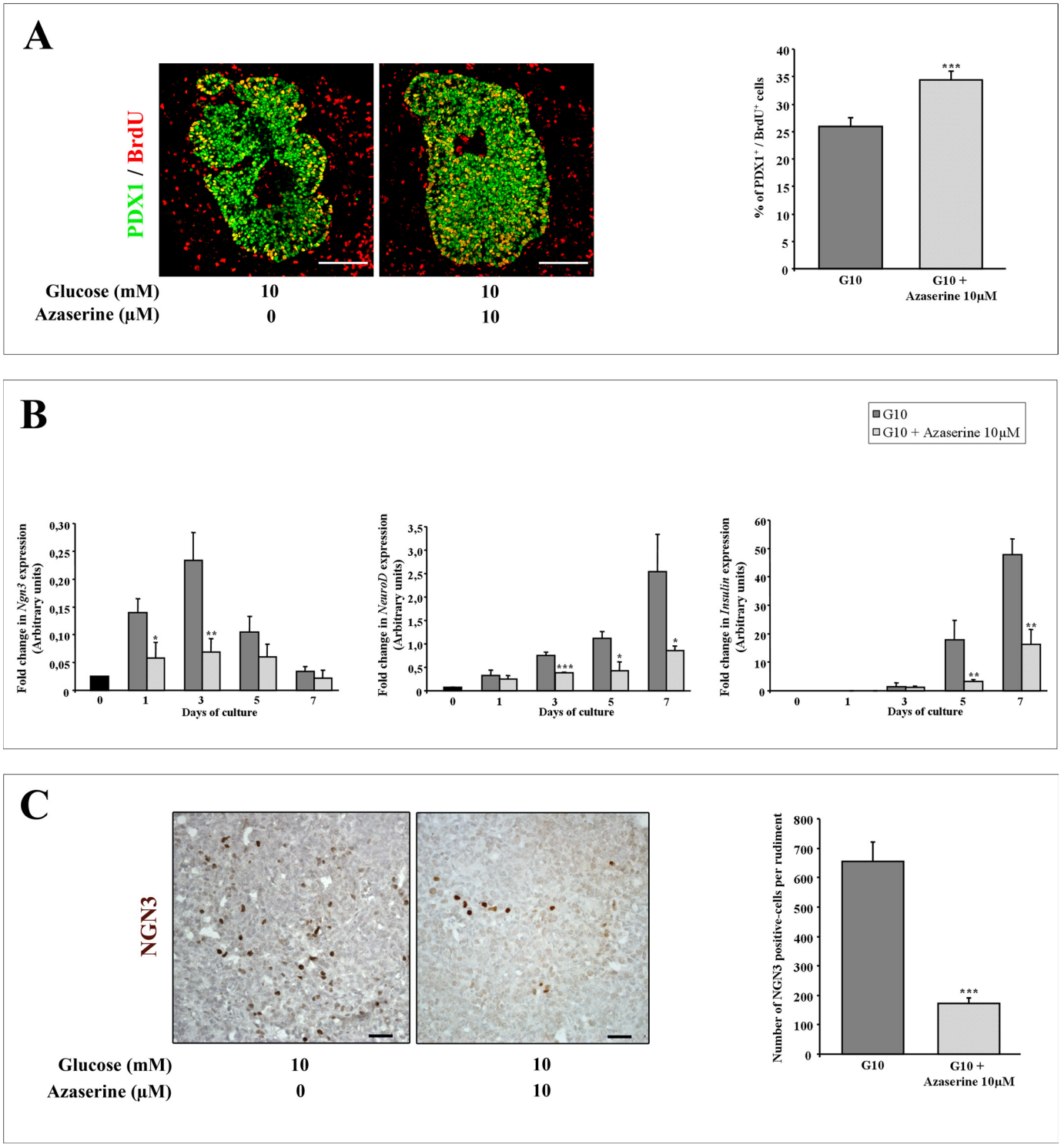
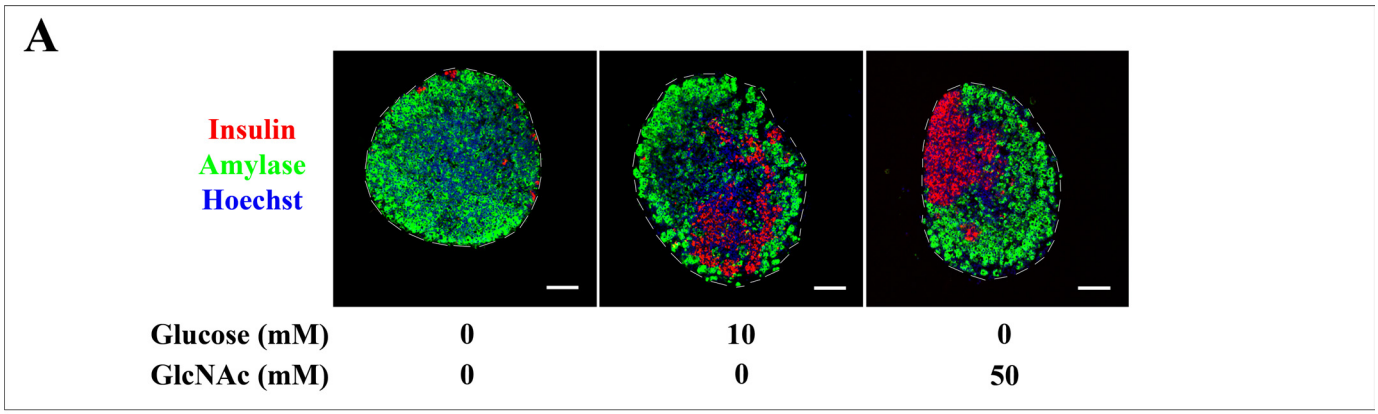
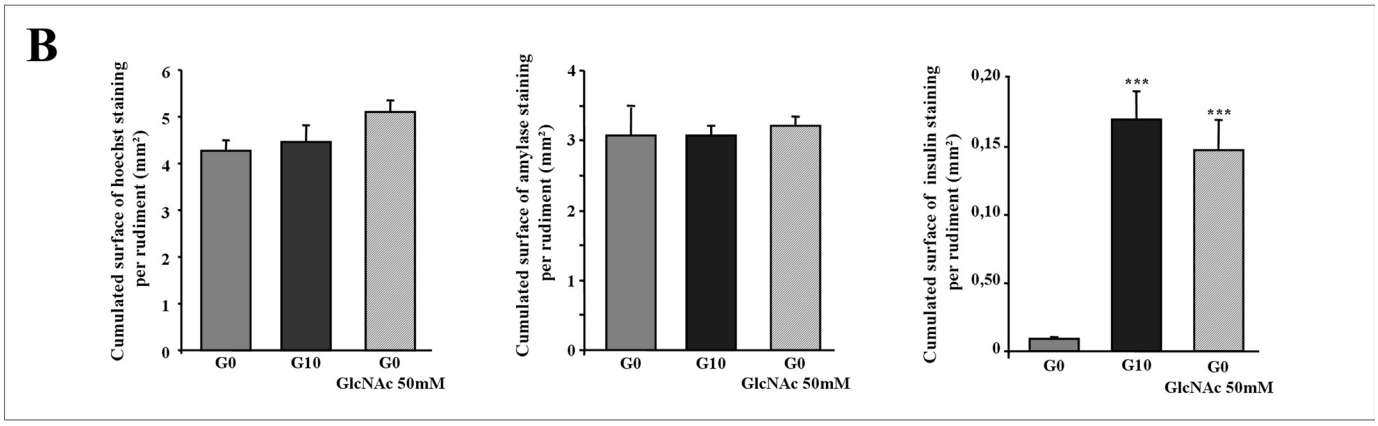
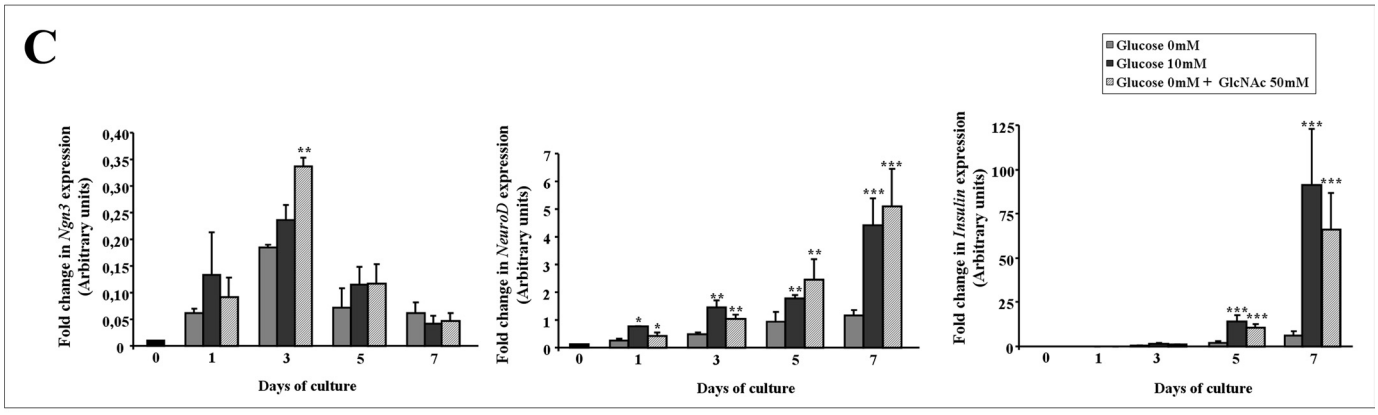


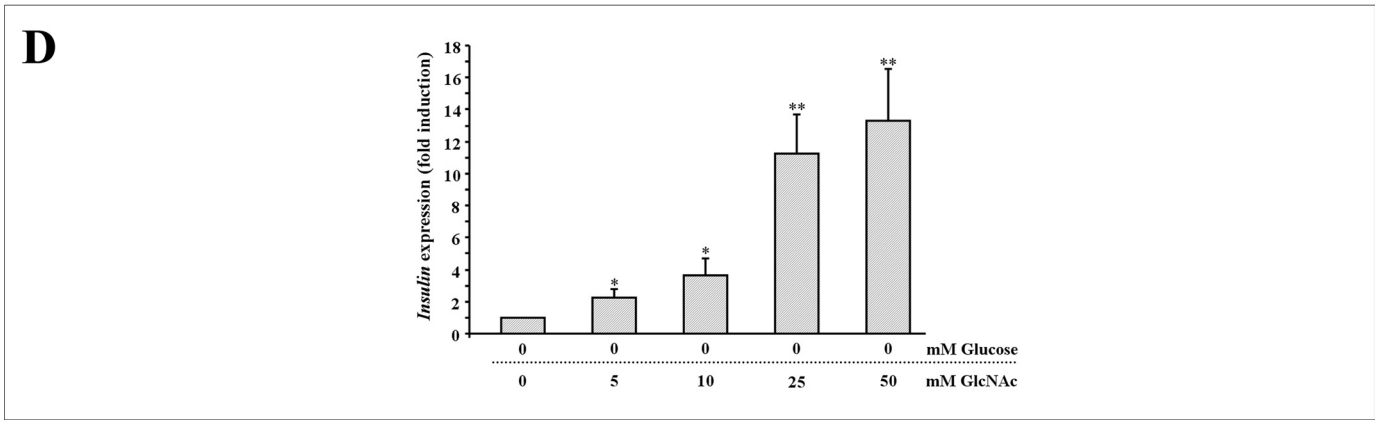
FIGURE 2. **Azaserine treatment prevents the development of NGN3-positive endocrine progenitor cells.** *A*, detection and quantification of proliferative PDX-1-positive pancreatic progenitors after culturing for 24 h in the presence of 10 mM glucose with or without azaserine. PDX-1- and BrdUrd-positive cells are stained *green* and *red*, respectively. Proliferating PDX-1-positive cells are shown in *yellow*. *Scale bar*, 100 μm. The values are the means ± S.E. of four independent experiments. ***, $p < 0.001$. *B*, real time PCR quantification of *Ngn3*, *NeuroD*, and *Insulin* mRNA in E13.5 pancreases before (Day 0) and after 1, 3, 5, and 7 days of culture in the presence of 10 mM glucose with or without azaserine. *C*, detection by immunohistochemistry and quantification of NGN3-positive endocrine progenitor cells in pancreases cultured for 5 days in the presence of 10 mM glucose with or without azaserine. *Scale bar*, 100 μm.

were cultured in the presence of 10 mM glucose with and without 10 μM azaserine. After 7 days of culture, azaserine treatment had not modified the size of the tissue (Fig. 1, *B* and *C*). However, it slightly but significantly decreased acinar cell differentiation as indicated by a 1.6-fold decrease in the surface area occupied by amylase-positive cells compared with control culture (Fig. 1, *B* and *C*). It also decreased *Ptf1a*, *Carboxypeptidase A*, and *Amylase* mRNA expression by 34, 47, and 41%, respectively (data not shown). Moreover, azaserine strongly decreased beta cell development, indicated by a 5-fold decrease


A
Insulin
Amylase
Hoechst
Glucose (mM) 0 10 0
GlcNAc (mM) 0 0 50


B
Cumulated surface of hoechst staining per rudiment (mm²)
G0 G10 G0
GlcNAc 50mM
Cumulated surface of amylase staining per rudiment (mm²)
G0 G10 G0
GlcNAc 50mM
Cumulated surface of insulin staining per rudiment (mm²)
G0 G10 G0
GlcNAc 50mM


C
Glucose 0mM
Glucose 10mM
Glucose 0mM + GlcNAc 50mM
Fold change in Ngn3 expression (Arbitrary units)
Days of culture
Fold change in NeuroD expression (Arbitrary units)
Days of culture
Fold change in Insulin expression (Arbitrary units)
Days of culture


D
Insulin expression (fold induction)
0 0 0 0 0 mM Glucose
0 5 10 25 50 mM GlcNAc

in the surface area occupied by insulin-positive cells compared with the control culture (Fig. 1, *B* and *C*). In addition, azaserine had a dose-dependent effect on beta cell development. Specifically, when pancreases were cultured for 7 days in the presence of 10 mM glucose at increasing concentrations of azaserine (from 0 to 10 μM), there was a progressive decrease in *Insulin* mRNA steady state levels as measured by real time PCR (Fig. 1*D*). Pancreatic endocrine and exocrine cells derive from early progenitors expressing the transcription factor PDX-1. A simple explanation for decreased development of both acinar and beta cell mass in the presence of azaserine could be that azaserine either induces apoptosis or reduces the proliferative rate of the PDX-1-positive progenitors. However, neither did azaserine treatment increase the rate of apoptosis as determined using the terminal deoxynucleotidyl transferase dUTP nick end labeling method (data not shown), nor did it decrease proliferation of Pdx-1-positive progenitors as determined after BrdUrd incorporation. In fact, proliferation of PDX-1-positive precursors increased slightly in the presence of azaserine (Fig. 2*A*).

Previously we showed that glucose controls beta cell differentiation by promoting the transition between Ngn3 and NeuroD (18). We then checked to see whether azaserine blocked such a transition between Ngn3 and NeuroD during beta cell development. To this end, we cultured pancreases with 10 mM glucose with and without 10 μM azaserine and used real time PCR to quantify *Ngn3*, *NeuroD*, and *Insulin* expression before treatment (day 0) and 1, 3, 5, and 7 days after treatment. At all time points analyzed, Ngn3 mRNA expression was significantly decreased in the presence of azaserine and remained low throughout the culture period (Fig. 2*B*). This decrease in mRNA was paralleled by a 3.8-fold decrease in the number of endocrine progenitors expressing the NGN3 protein (Fig. 2*C*). Consequently, expression of downstream targets such as *NeuroD*, *Insulin* (Fig. 2*B*), and *MafA* and *MafB* (data not shown) was dramatically decreased after treatment with azaserine (about 60% decrease for *NeuroD*, 66% for *Insulin*, 47% for *MafA*, and 40% for *MafB*).

Collectively, these results indicate that complete inhibition of the HBP blocks beta cell development in the presence of glucose. Because azaserine acts on the HBP upstream of Ngn3, it was not possible with this protocol to determine whether HBP controls the transition between Ngn3 and NeuroD.

GlcNAc Mimics the Effect of Glucose on Beta Cell Differentiation—To further delineate the role of HBP in beta cell differentiation, we compared the effects of glucose and GlcNAc, which is a direct substrate of the HBP, on pancreatic development in E13.5 pancreases after 7 days in culture. Neither glucose nor GlcNAc affected pancreatic growth or acinar cell development (Fig. 3, *A* and *B*). Interestingly, GlcNAc mimicked the inductive effect of glucose on beta cell development; specifically, 15 times more insulin-positive cells developed after GlcNAc exposure compared with cultures grown in the absence of glucose (Fig. 3, *A* and *B*). To rule out the possibility that effect of GlcNAc was due to an osmotic change, we compared the effect of 50 mM GlcNAc to 50 mM L-glucose, a non-transported sugar. After 7 days of culture in the presence of such sugars, *Insulin* and *Amylase* expression were analyzed by real time PCR and compared with pancreases developed in the absence of added glucose. *Amylase* expression remained unchanged in all culture conditions, whereas *Insulin* expression was induced by 50 mM GlcNAc but not by 50 mM L-glucose (data not shown).

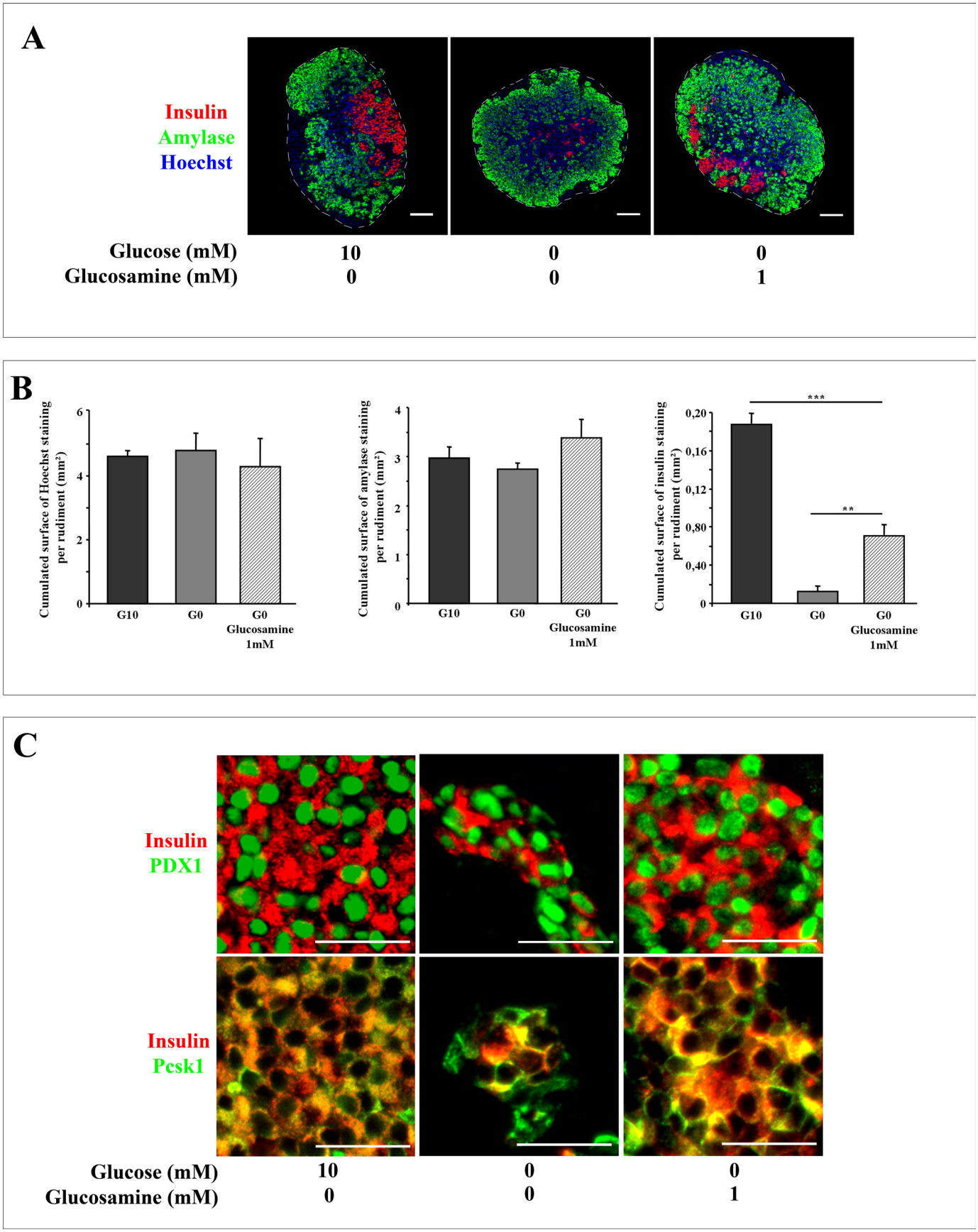
Next, to determine whether GlcNAc and glucose control the same step of pancreatic differentiation, we cultured pancreases and analyzed, using real time PCR, expression of *Ngn3*, *NeuroD*, and *Insulin* before exposure (day 0) and after 1, 3, 5, and 7 days in culture with and without 10 mM glucose and 50 mM GlcNAc. Overall, the expression profile of *Ngn3* was similar in all culture conditions. Interestingly, the *Ngn3* mRNA steady state level was slightly but significantly increased at day 3 of culture with GlcNAc compared with the other culture conditions (Fig. 3*C*). Similarly, the number of NGN3-positive cells was also higher on day 3 of GlcNAc culture (data not shown). Importantly, both glucose and GlcNAc induced an increase in the level of the NGN3-target *NeuroD* (Fig. 3*C*). Consequently, glucose and GlcNAc also induced *Insulin* expression (Fig. 3*C*).

When embryonic pancreases were cultured for 7 days with GlcNAc at concentrations ranging from 0 to 50 mM, real time PCR showed that the *Insulin* mRNA level significantly increased in a dose-dependent manner with increasing concentrations of GlcNAc (Fig. 3*D*). However, there was no concurrent effect on acinar cell development (Fig. 3, *A* and *B*, and data not shown), indicating that activation of the HBP only selectively affected pancreatic endocrine cell development.

Glucosamine Reproduces the Effect of Glucose on Beta Cell Differentiation—GlcNAc is not only a substrate of the HBP, but also a hexokinase activity inhibitor (27). Thus, to validate that the positive effect of GlcNAc on beta cell development was due to a specific activation of the HBP, we compared the effects of glucose and glucosamine, a specific substrate of the HBP pathway (Fig. 1*A*) (28), on the beta cell differentiation process. After 7 days of culture, neither glucose nor glucosamine (1 mM) affected pancreatic size or acinar cell differentiation (Fig. 4, *A* and *B*). However, both glucose and glucosamine increased beta cell development (Fig. 4, *A* and *B*). Such beta cells developed with glucosamine express many of the same markers found in mature beta cells, such as the transcription factor PDX-1 and the proconvertase PCSK1 (Fig. 4*C*), as was the case with GlcNAc (data not shown).

Inhibition of O-GlcNAc Transferase Decreases Beta Cell Development—The HBP promotes *O*-glycosylation of proteins by OGT (29). Because BADGP is an inhibitor of OGT, we tested

FIGURE 3. **GlcNAc is sufficient to induce beta cell differentiation in the absence of glucose.** E13.5 rat pancreases were cultured in the absence (G0) or presence of 10 mM glucose (G10) or in the absence of glucose plus 50 mM GlcNAc (G0 + GlcNAc). *A*, immunohistological analysis of pancreases after 7 days of culture. Insulin is revealed in *red*, and amylase is in *green*. The nuclei are depicted in *blue* with Hoechst stain. *Scale bar*, 100 μm. *B*, quantification of the absolute surface area occupied by Hoechst-, amylase-, and insulin-positive cells after 7 days of culture with or without GlcNAc. *C*, real time PCR quantification of *Ngn3*, *NeuroD*, and *Insulin* mRNA in E13.5 pancreases before (day 0) and after 1, 3, 5, and 7 days of culture. *D*, real time PCR quantification of *Insulin* mRNA after 7 days of culture in the absence of glucose (G0) and at increasing concentrations of GlcNAc. The values are the means ± S.E. of five independent experiments and were compared with the G0 condition. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.


A
Insulin
Amylase
Hoechst
Glucose (mM) 10 0 0
Glucosamine (mM) 0 0 1
B
Cumulated surface of Hoechst staining per rudiment (mm²)
Cumulated surface of amylase staining per rudiment (mm²)
Cumulated surface of insulin staining per rudiment (mm²)
G10
G0
G0 Glucosamine 1mM
***
**
C
Insulin
PDX1
Insulin
Pcsk1
Glucose (mM) 10 0 0
Glucosamine (mM) 0 0 1

its effect on pancreatic cell development. Pancreases were cultured with 10 mM glucose alone or supplemented with 5 mM BADGP for 7 days, and acinar and beta cell differentiation was analyzed immunohistochemically. BADGP treatment did not affect either the size of the tissue or acinar cell development, but it caused a 2-fold decrease in beta cell development (Fig. 5, *A* and *B*). Next, we analyzed by real time PCR the expression of *Ngn3*, *NeuroD*, *Insulin*, *MafA*, and *MafB* before treatment (day 0) and after 1, 3, 5, and 7 days in culture with 10 mM glucose alone or supplemented with BADGP, to determine which step in the beta cell differentiation process was affected by BADGP. The addition of BADGP treatment did not modify *Ngn3* expression (Fig. 5*C*), but it decreased expression of both *NeuroD* and *Insulin* (Fig. 5*C*). *MafA* and *MafB* expression was reduced by 46 and 67%, respectively (data not shown). To further support the results obtained with BADGP, we repeated the experiments with ST04584, another OGT inhibitor. Treatment with ST045849 at 20 μM for 7 days mimicked the effects observed with BADGP; neither *Ngn3* expression nor acinar cell development were modified upon ST045849 treatment (Fig. 5*C*, and data not shown), whereas *NeuroD* and *Insulin* expression decreased (Fig. 5*C*). Thus, cultures treated with glucose plus OGT inhibitors gave rise to a phenotype resembling the one found in cultures grown in the absence of glucose.

Inhibition of O-GlcNAcase Increases Beta Cell Development—Thus far, our results indicate that inhibition of OGT blocks beta cell differentiation after *Ngn3* activation but before *NeuroD* is targeted. Protein *O*-glycosylation by OGT is counteracted by the deglycosylating enzyme *O*-GlcNAcase (28). Therefore, to confirm that *O*-glycosylation plays a role in beta cell differentiation, we tested whether treatment with PUGNAc, an inhibitor of *O*-GlcNAcase, activates beta cell development. We cultured pancreases for 7 days in the absence of added glucose with and without 10 μM PUGNAc. PUGNAc treatment did not affect the size of the tissue or acinar cell differentiation, but it increased beta cell mass 3.5-fold (Fig. 6, *A* and *B*). We then analyzed expression of *Ngn3*, *NeuroD*, *Insulin*, *MafA*, and *MafB* using real time PCR, before (day 0) and after 1, 3, 5, and 7 days in culture to determine how PUGNAc treatment affects beta cell development. PUGNAc did not modify *Ngn3* expression, but it induced *NeuroD* and *Insulin* expression (Fig. 6*C*). *MafA* and *MafB* expression was also induced by 3.6- and 4.4-fold, respectively (data not shown). However, PUGNAc did not increase *Insulin* expression to the same degree as treatment with glucose alone. In fact, even when the culture medium is not supplemented with glucose, the glucose concentration in the medium is ~1 mM because of the addition of fetal calf serum. Consequently, we compared the effect of PUGNAc alone and in combination with glucose at concentrations of 1, 2, and 10 mM. As shown in Fig. 6*D*, increasing glucose concentrations enhanced the effects of PUGNAc on beta cell development.

DISCUSSION

Pancreatic development during prenatal life is a tightly regulated process, and many signals have been implicated in its control (reviewed in Ref. 30). Specifically, pancreas development is initially controlled by fibroblast growth factor 2 (FGF2) and activin βB produced by the notochord (12, 31), then by blood vessel-derived sphingosine-1-phosphate (13), and finally by FGF10 produced by the pancreatic mesenchyme (32, 33). Less is known regarding the role of environmental signals and nutritional environment on pancreatic cell development. Data derived from numerous epidemiologic studies (34, 35) and animal studies using models of intrauterine growth restriction (16, 36–41) have established a link between the intrauterine nutritional environmental status, pancreas development, and the increased risk for developing type 2 diabetes (42). However, none of these studies were designed to define the role of specific nutrients and to dissect their function at the cellular level.

We have established an *in vitro* model using embryonic rat pancreases that mimics the major developmental steps occurring *in vivo* (17). With this model, we have found that glucose does not affect cell proliferation or acinar cell development but does control major steps of endocrine cell development. Specifically, the first steps of endocrine cell development, such as proliferation of PDX-1-positive pancreatic progenitor cells and induction of NGN3-positive endocrine progenitors, were glucose-independent. On the other hand, induction of *NeuroD*, a NGN3 target necessary for proper beta cell development (8, 10), did not occur in the absence of glucose, giving rise to poor beta cell development (18). These effects of glucose, obtained using dorsal pancreatic explants, were also observed using ventral pancreatic explants (data not shown).

Here, to dissect how glucose positively controls pancreatic endocrine cell development, we investigated the implication of glucose metabolism and more precisely the role of the HBP (43). This pathway diverts 2–5% of the fructose-6-phosphate derived from glucose into glucosamine-6-phosphate, giving rise to substrates for the synthesis of glycoproteins and glycolipids (19). To search for a role of HBP in pancreatic development, we analyzed the effect of the following series of HBP inhibitors and activators. Azaserine, an inhibitor of glutamine amidotransferases, was used to inhibit GFAT (43, 44), the first rate-limiting enzyme in HBP that catalyzes an essential irreversible reaction (19). The effects of glucosamine and GlcNAc, which are substrates of glucosamine-6-P and uridine diphosphate-GlcNAc (44), respectively, were also analyzed. Finally, we used BADGP and PUGNAc, which are negative and positive regulators of *O*-linked glycosylation, respectively (45, 46).

Our approach is based on known activators and inhibitors of the HBP. This strategy of using HBP inhibitors and activators has been successfully used in many cells types and organs to

FIGURE 4. **Glucosamine mimics the effect of glucose on beta cell differentiation.** *A*, immunohistological analysis of pancreases after 7 days of culture in the absence or presence of glucose or in the absence of glucose plus 1 mM glucosamine. Insulin is revealed in *red*, and amylase is in *green*. The nuclei are depicted in *blue* with Hoechst stain. *Scale bar*, 100 μm. *B*, quantification of the absolute surface area occupied by Hoechst-, amylase-, and insulin-positive cells after 7 days of culture with or without 1 mM glucosamine. The values are the means ± S.E. of five independent experiments and were compared with the G0 condition. **, $p < 0.01$; ***, $p < 0.001$. *C*, immunohistological analysis of mature beta cell markers after 7 days of culture with or without 1 mM glucosamine. Insulin is revealed in *red*, and PDX-1 (or Pcsk1) is in *green*. *Scale bar*, 25 μm.

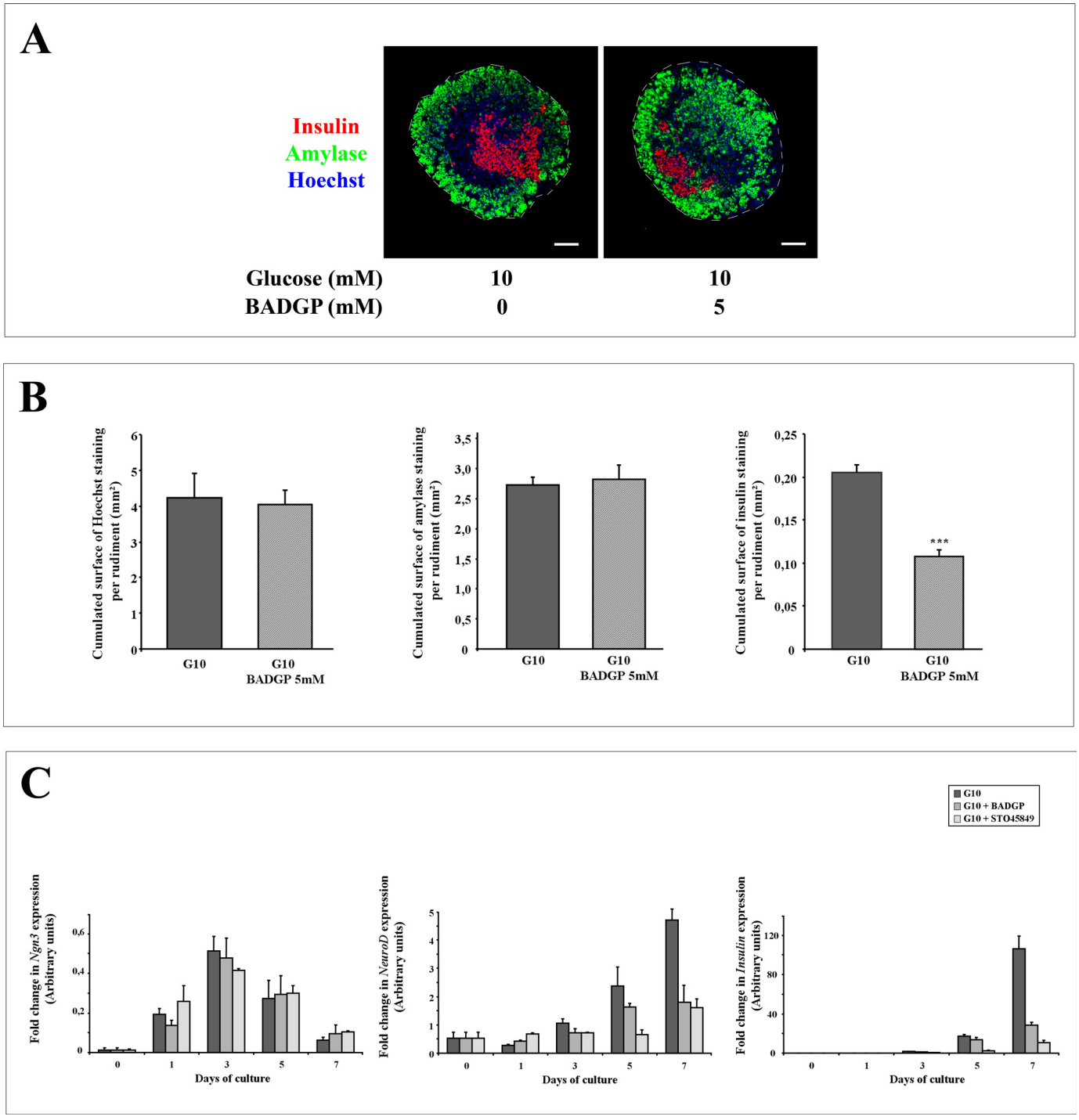


FIGURE 5. **OGT inhibitors treatment reduces beta cell development.** *A*, immunohistological analysis of pancreases after 7 days of culture in the presence of 10 mM glucose with or without 5 mM BADGP. Insulin is revealed in *red*, and amylase is in *green*. The nuclei are depicted in *blue* with Hoechst stain. *Scale bar*, 100 μm. *B*, quantification of the absolute surface area occupied by Hoechst-, amylase-, and insulin-positive cells after 7 days of culture with or without 5 mM BADGP. *C*, real time PCR quantification of *Ngn3*, *NeuroD*, and *Insulin* mRNA in E13.5 pancreases before (day 0) and after 1, 3, 5, and 7 days of culture in the presence of 10 mM glucose, with or without 5 mM BADGP, or with or without 20 μM ST045849. The values are the means ± S.E. of at least three independent experiments and were compared at each time point to the culture not supplemented with an OGT inhibitor. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

dissect the role of this pathway. For example, in the kidney, this approach was used to demonstrate that HBP is involved in glucose-induced mesangial production of transforming growth factor β, leading to increased matrix production (47). In the liver, HBP activators and inhibitors were used to show how HBP triggers hepatic gluconeogenesis (48). In addition, this strategy demonstrated that HBP is involved in the development of insulin resistance in adipocytes (49). With regard to its role in the pancreas, it was recently demonstrated that HBP regulates the function of two transcription factors implicated in beta cell function. Specifically, glucose induces expression of the beta cell-specific transcription factor MafA via the HBP (50). In addition, Andrali *et al.* (51) reported that glucose mediates the translocation of NeuroD by *O*-linked glycosylation. To the best

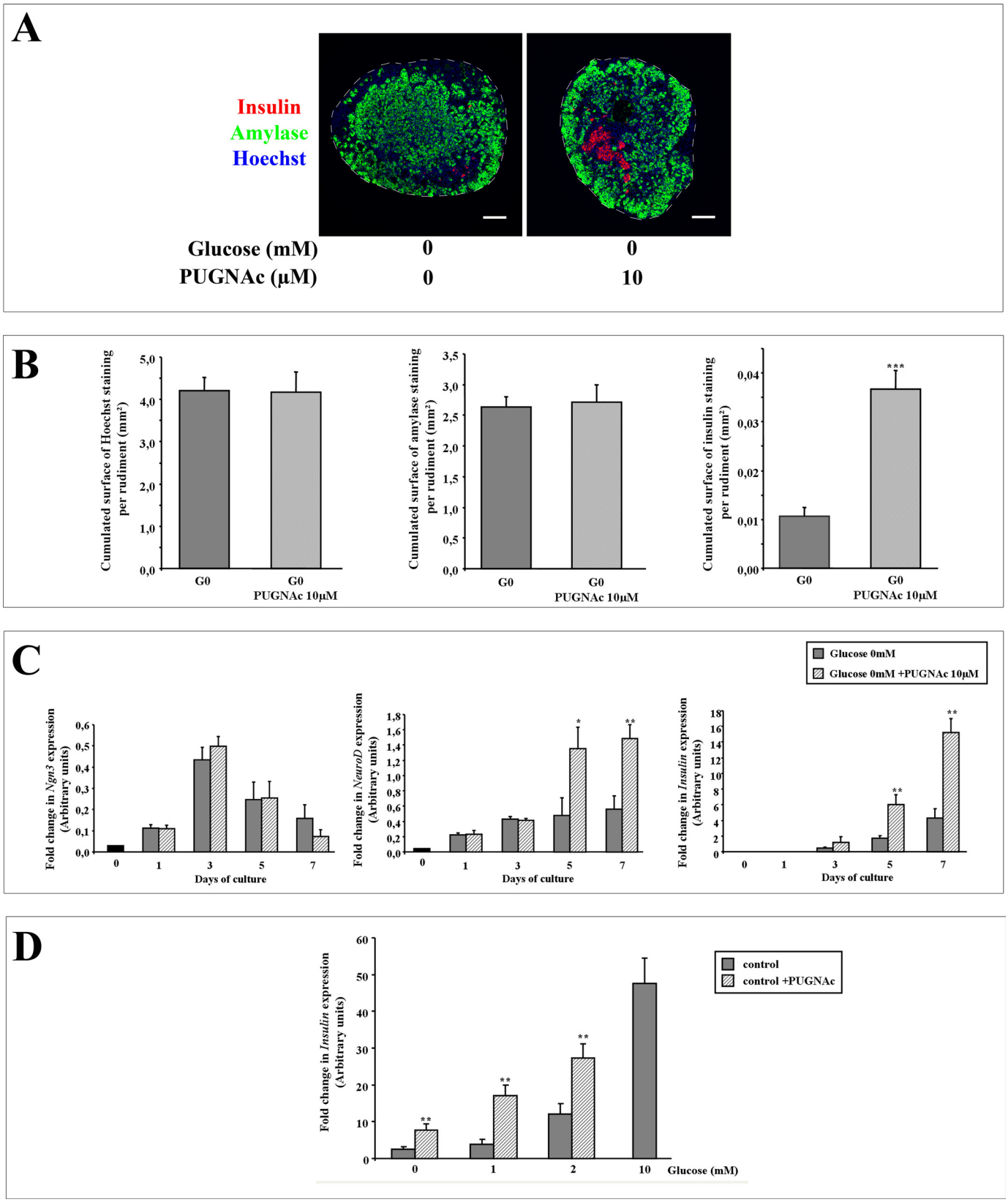


FIGURE 6. **PUGNAc treatment improves beta cell development.** *A*, immunohistological analysis of pancreases after 7 days of culture without glucose and with or without 10 μM PUGNAc. Insulin is revealed in *red*, and amylase is in *green*. The nuclei are depicted in *blue* with Hoechst stain. *Scale bar*, 100 μm. *B*, quantification of the absolute surface area occupied by Hoechst-, amylase-, and insulin-positive cells after 7 days of culture with or without 10 μM PUGNAc. *C*, quantification by real time PCR of *Ngn3*, *NeuroD*, and *Insulin* mRNA in E13.5 pancreases before (day 0) and after 1, 3, 5, and 7 days of culture in the absence of glucose or in a medium containing PUGNAc 10 μM without glucose. Comparisons were performed at each time point between the two conditions. The values are the means ± S.E. of five independent experiments. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. *D*, real time PCR quantification of *Insulin* mRNA after 7 days of culture in the presence of increasing concentrations of glucose with or without 10 μM PUGNAc. Comparisons were performed between the control and treated cultures.

of our knowledge, there are no data on the role of the HBP in pancreatic cell development. Although the nonspecific effects of activators and inhibitors of the HBP pathway cannot be fully excluded, it is of importance to note that treatment with inhibitors did not modify ATP content or the indexes of glucose utilization rate (data not shown). Moreover, the different substrates or inhibitors used in the present study that act on different targets give results that fit perfectly with the proposed model.

Proper pancreatic cell development depends on the signals controlling cell proliferation and differentiation (32, 33, 52, 53). In the series of experiments we report here, pancreatic cell growth was not modified, indicating that the HBP does not play a major role in pancreatic cell proliferation during embryonic life. These data support our previous findings that glucose does not modify pancreatic cell proliferation during this period of embryonic development (18). Moreover, at this stage, pancreatic cell proliferation is mainly dependent on FGF receptor 2b ligands such as FGF10, which acts on early Pdx-1-positive pancreatic progenitors (17). Interestingly, in this study, azaserine affected cell proliferation by increasing the proliferative rate of PDX-1-positive pancreatic progenitors. The question of whether HBP enhances the FGF10-FGF 2b pathway has yet to be studied.

Perturbation of the HBP had only a minor effect on acinar cell differentiation. Exposure to glucosamine, GlcNAc, BADGP, and PUGNAc did not alter acinar cell development. Regardless of the culture condition, the same number of acinar cells developed. Again, these results support our previous findings that acinar cells develop in a glucose-independent manner (18). However, our results showed that azaserine treatment induced a small but reproducible decrease in acinar cell development. As indicated above, this cannot be explained by a decrease in the number of PDX1-positive progenitors but could be linked to an effect of azaserine on specific transcription factors implicated in acinar cell differentiation such as Ptf1a (54) or Mist1 (55).

When the HBP was blocked, beta cell differentiation decreased, whereas the addition of HBP substrates increased beta cell differentiation. Beta cell differentiation depends on a cascade of events controlling the expression of a series of transcription factors at specific time points (56, 57). Knowledge of this well characterized cascade has permitted investigators to define factors and conditions controlling specific steps of pancreatic development under proper experimental systems (13, 17, 26, 31). Here, we demonstrated that all the treatments used to perturb the HBP modified beta cell development, mimicking phenotypes previously observed when glucose concentrations were modified (18). Moreover, the effects of treatment with glucosamine, BADGP, and PUGNAc closely mimicked those of glucose by controlling the same step of development (*i.e.* acting between Ngn3 and its target NeuroD). In our study it was not possible to determine whether azaserine acted downstream of Ngn3 because azaserine exposure had already decreased *Ngn3* expression. Interestingly, GlcNAc treatment induced a reproducible increase in *Ngn3* expression (Fig. 3*C*). There is little information on the control of *Ngn3* expression, other than from signals derived from the Notch pathway (58). Azaserine is an amidotransferase inhibitor that inhibits other amidotransferases in addition to GFAT. Further studies must be conducted to determine whether Ngn3 expression is controlled by HBP or by other aminotransferases.

HBP is involved in protein *N*- and *O*-glycosylation. *O*-Glycosylation is a common post-translational modification that is implicated in numerous cellular processes like activation of transcription factors (59), the nucleocytoplasmic shuttle (20), or cell development (60, 61). Our data using inhibitors controlling *O*-glycosylation suggest that an *O*-glycosylated factor favors the transition between Ngn3 and NeuroD. We are now defining strategies to characterize the nature of such an *O*-glycosylated factor, controlling *NeuroD* expression.

Establishment of controlled conditions that permit efficient generation of functional beta cells from embryonic stem cells is challenging. Although there has been important progress in this field (62), information is missing for human beta cell development in controlled conditions. The present work provides new information on pancreas development and reports the inductive effect of the HBP on pancreatic beta cell differentiation.

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